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The spatial distribution of the EGF receptor and ligands is necessary for normal organization and proliferation of human mammary epithelial cells. We found that these cells express high levels of EGF receptors. These receptors are rapidly internalized and recycled. Unlike transformed cells, internalization efficiency of normal HMEC is high. These cells also make high levels of EGF, TGF-alpha, HB-EGF and amphiregulin, although TGF-alpha and amphiregulin appear to be the predominant ligands. We transfected cells with a gene encoding an artificial ligand consisting of the mature sequences of EGF (sEGF). Cells expressing sEGF could not be inhibited by antagonistic anti-EGF-R antibodies, apparently because the sEGF was working through an intracrine mechanism. Importantly, cells expressing sEGF could not organize correctly. Also, the ability of HMEC to spread and migrate on the extracellular matrix was dependent of EGF-R occupancy. Our data suggests that the ability to HMEC to spatially sense their environment is dependent on the EGF-R system through an autocrine mechanism. Importantly, these data support our hypothesis that disruptions in the spatial aspects of EGF-R signaling could facilitate the development of breast cancer.

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INTRODUCTION

An important goal of current breast cancer research is to develop an in vitro system that can define the mechanisms involved in the progression of human mammary epithelial cells (HMEC) towards a transformed phenotype. In this project, we have focused on an aspect of HMEC behavior that is likely to be involved in this progression, namely, the correct spatial sorting of growth factors and their receptors to discrete cellular locations. We chose this research focus for two important reasons: 1) recent evidence indicates that defects in receptor/ligand trafficking is a hallmark of proliferative disorders in epithelial cells (1,2), and 2) since receptor trafficking is primarily a negative regulatory process defects in this pathway are likely to amplify receptor signaling (3,4). Because correct receptor trafficking depends on the function of many intracellular regulatory systems, it provides a sensitive readout of their status. The EGF receptor system is used as the primary experimental model because it plays a central role in the growth, motility and proliferation of normal HMEC as well as many breast cancers (5-8). Therefore any significant alterations in growth factor regulation in HMEC is likely to perturb the EGF receptor system.

The functions of growth factors extend far beyond simple growth regulation. They are involved in cell differentiation, chemotaxis, morphogenesis, wound healing and gastric acid secretion (9). Originally, growth factors were thought to be products secreted by cells, but in fact, many are produced as membrane-associated precursors. For example, EGF is initially produced as a 170 kDa membrane protein (10) and transforming growth factor alpha (TGF-α) is produced as a 20-22 kDa MW precursor (11). In the case of TGF-α, release from the cell surface occurs through regulated proteolysis (12). The multiple levels at which availability of growth factors can be regulated provide many opportunities for fine control of tissue functions.

Three main routes of growth factor signaling are currently recognized: autocrine, paracrine and juxtacrine (13). In autocrine signaling, cells make both the growth factor and the complementary receptors. In general, the factors must be transported to the cell surface to be functional. In paracrine signaling, different cells make the ligand and receptors. The factor must be transported from the site of production to the site of binding, usually by diffusion. Finally, juxtacrine signaling occurs when receptors on one cell bind directly to the membrane-associated ligand on another cell. All of these types of signaling can be regulated by controlled synthesis, rate of ligand release, and by competition for ligand capture either between different cells or by extracellular matrix proteins (13). Growth factor signaling is also regulated by the physical separation of the ligand and receptor at the cell surface or within the endocytic pathway. This spatial regulation is mediated by sorting components which bind to receptor cytoplasmic domains (14). Growth factors may also be synthesized initially as transmembrane proteins, presumably allowing cells to physically segregate them from receptors.

Epithelial cells display a high degree of spatial organization as evidenced by their polarized phenotype. Kidney, breast and intestinal epithelial cells all show similar features; all are associated through tight junctions and have distinct basolateral and apical surfaces (15). In-vivo, breast epithelium is organized into ducts, ductules and alveoli consisting of a basement membrane, a discontinuous layer of myoepithelial cells, a layer of basal epithelial cells and a layer of luminal cells (16). Both basal and luminal cells display a polarized distribution of integrins and EGF-R (17-19). Integrins mediate interactions with the basement

membrane and appear essential for controlling specific gene expression and maintaining polarization and differentiated functions (20,21). EGF-R are important in regulating epithelial cell growth, and in the breast, are expressed at high levels in myoepithelial cells, basal cells and at the basolateral surface of luminal epithelial cells (19). The functional significance of the basolateral distribution of these receptors is not understood, but could be involved in maintaining the correct organization of epithelial cells within tissues.

Three ligands are thought to be produced in mature breast alveoli which can bind to the EGF-R: EGF, TGF-α and amphiregulin (5,22,23). The best studied of these, TGF-α, is produced by epithelial cells and at least in the mouse, is localized at their basolateral surface (24). Because the basolateral surface contains the EGF-R, the space between this surface and the basement membrane comprises the "microenvironment" in which signaling through the EGF-R occurs. Although EGF is found at high concentrations in the ductal lumen of both mouse and human, little TGF-α is found in breast milk or nipple aspirates of humans (approximately 0.8 ng/ml and 5 ng/ml respectively), indicating a polarized secretion of EGF to the apical surface and TGF-α to the basolateral surface of luminal cells (25). Nothing is known regarding the distribution of amphiregulin in HMEC, but in intestinal cells it displays a luminal distribution (26). Significantly, an extremely high concentration of EGF is found in breast fluids of non-lactating (>200ng/ml) or lactating (100-140 ng/ml) women (25). These concentrations are 2 orders of magnitude higher than the K_d of the EGF-R in HMEC. Therefore, the polarized organization of HMEC segregates their EGF-R from a large reservoir of active hormone.

Very little is known regarding the loss of HMEC polarity during transformation. Current data suggest that integrins (such as α6/β4) retain their polar distribution during early phases of proliferative diseases, but this organization is lost in poorly differentiated invasive tumors (17,27). However, several lines of evidence suggest that the polarized distribution of integrins is a consequence of their binding to the extracellular matrix or to cellular neighbors and is not directly regulated by the cell (18). In contrast, there are a number of reports on the loss of EGF-R polarity in breast cancer . Significantly, oncogene expression can cause a redistribution of selective membrane components in epithelial cells. For example, K-*ras* expression in MDCK cells converts a monolayer of polarized cells to a multilayer in which selective apical proteins appear on the basolateral surface (28). In polycystic kidney disease, there is a reversal of Na/K ATPase (29) and EGF-R localization (30), but distribution of most of the other membrane proteins is unaffected.

Correct spatial organization of epithelial cells is necessary for their differentiated functions (secretion of milk proteins, etc.). Growth factors are available only from specific locations and contact with the basement membrane is required for proliferation (15,21). This imposes regulatory constraints on epithelial cells that are probably essential for maintaining structural homeostasis.

A great many studies have investigated the relationship between EGF-R and breast cancer (8,19,31,32). In general, overexpression of the EGF-R in breast tumors indicates poor prognosis, but other growth factor receptors, such as HER2/*neu*, also appear to be linked to breast cancer (8). So why study the physiology of the EGF-R? There are several compelling reasons. The incidence of overexpression of the EGF-R is more common than overexpression of HER2/*neu* (45% versus 20% respectively; (8)). Significantly, less than 20% of the tumors

that display overexpression of the EGF-R also show amplification of the EGF-R gene, whereas all incidents of HER2/*neu* overexpression appear to be due to gene amplification (33). This indicates that the EGF-R is subject to multiple levels of control that can be independently altered during transformation. Tight control on the EGF-R system is probably necessary because it appears to be the major regulator of HMEC proliferation in vivo. EGF-containing pellets can stimulate normal ductal growth in regressed mammary glands of ovariectomized mice (24). Estrogens appear to regulate the proliferation of HMEC in vivo and in vitro in part through an EGF-R autocrine loop (34,35). Blocking EGF-R occupancy in vitro using a monoclonal antibody causes HMEC to reversibly enter G_0 (6). EGF is essential for the motility and assembly of HMEC into organized alveolar structures in vitro. EGF also has a dual effect of promoting growth and chemotaxis/motility of keratinocytes (36) and intestinal epithelial cells (37), suggesting that it has a general role in both establishing and maintaining the structure of epithelial tissues.

Because of the importance of the EGF-R in HMEC regulation, it appears likely that genetic alterations that give HMEC a growth advantage will operate either directly or indirectly through this receptor system. Despite the numerous studies on EGF-R and breast cancer, this idea has not been critically tested. Studies that document the presence or absence of EGF-R (or their overexpression) are not particularly informative in this regard. For example, the MCF-7 breast cancer cell line displays very low levels of EGF-R expression compared to normal HMEC, but estrogen can induce proliferation in these cells in part through an EGF-R/TGF-α autocrine pathway (38). In rapidly proliferating HMEC, there is a positive relationship between TGF-α levels and proliferation, apparently due to a positive feedback loop operating through the EGF-R (5). Amphiregulin and EGF-R levels are also high in proliferating HMEC, but not in intact organoid structures (5,23). Control of receptor number could regulate other aspects of HMEC function, such as directional sensing of ligands. In addition, genetic lesions that operate downstream of the EGF-R itself would not necessarily affect receptor expression. The present uncertainty regarding the role, if any, of the EGF-R in breast cancer reflects our general lack of understanding of its role in normal epithelial cell function, an issue directly addressed by our studies.

By its very nature, cancer is a dysfunction in cellular organization and regulation. Premalignant cells are likely to display specific defects that allow for their clonal expansion. Genetic lesions that facilitate the progression to breast cancer could operate either upstream or downstream of the EGF-R system . The upstream lesions could result in inappropriate spatial organization or expression of either the EGF receptor or its ligands, facilitating cell proliferation and/or motility. Downstream lesions could constitutively activate signal transduction pathways or genetic programs normally triggered by EGF-R occupancy. This would result in the expression of an EGF "response" in the absence of the ligand. Defects in upstream intracellular trafficking pathways or downstream signal transduction pathways may not be specific to the EGF-R. However, any generalized cellular changes will be reflected in alterations in the pathways followed by the EGF-R or the responses that it triggers. To identify premalignant lesions, we must establish a detectable phenotype. An investigation of the EGF-R system in HMEC is likely to both establish such a phenotype and improve our understanding of normal HMEC physiology.

BODY

Our original hypothesis was that loss of normal polarized distribution of EGF-R provides a significant growth advantage to affected cells by allowing access to high concentrations of growth factors normally found in luminal fluids. There were four major tasks listed in the Statement of Work:

> Task 1: Define the Changes That Occur in the Spatial Organization of Both the EGF-R and its Ligands During Formation of Organized Alveolar Structures in Vitro (Months 1-24)
>
> Task2: Determine Whether a Loss in the Correct Spatial Organization of the EGF Receptor is Associated with a Loss of Specific Tumor Suppressor Genes (Months 12-36)
>
> Task 3: Express genetically altered EGF receptors in mammary epithelial cells (Months 12-36)
>
> Task 4: Demonstrate That Mis-sorting or Inappropriate Expression of the EGF Receptor or its Ligands Provides a Growth Advantage to Normally Organized Epithelial Cells (Months 24-48)

We have made excellent progress in the last year, particularly with respect to tasks 1 and 3. Progress on task #2 has been partially slowed by difficulties in identifying an appropriate model cell system. For example, we found that the cells we originally proposed to use to examine the spatial organization of the EGF receptor (184A1) was not the best cell type for those studies. The difficulty was that cells did not make tight enough junctions to allow simple discrimination of apical/basolateral distribution of membrane proteins. Recently, we have obtained the more suitable HB2 cells from Dr. Joyce Taylor-Papadimitiou. These cells are human mammary epithelial cells derived from milk and immortalized with SV40 large T antigen. These cells possess a luminal phenotype (characteristic of malignant mammary epithelial cells), but are non-tumorgenic. Importantly, they express high numbers of EGF receptors and form well polarized monolayers. These cells appear to be ideal for the studies outlined in task 2 and we expect significant progress on that aspect in the next year.

The rest of this report is organized around the specific subtasks of Task 1 because that was the only task that was scheduled to be done in the first year.

A. The in situ localization methodology for the EGF receptor and its ligands will be refined.

We have made excellent progress in refining our immunolocalization techniques for examining the EGF receptor. For example, Figure 1 shows the effect of EGF addition on the distribution of the EGF-R in HMEC. Note that the EGF-R is distributed both at the cell surface as well as intracellular in the absence of ligand. However, following the addition of EGF, there is a dramatic loss of EGF-R from the cell surface and a redistribution to the perinuclear area of the cell. In addition, there is an overall loss of EGF-R mass from the cells. Current data suggests that this redistribution is due primarily to an increased endosomal retention and lysosomal targeting of the occupied EGF-R (see below).

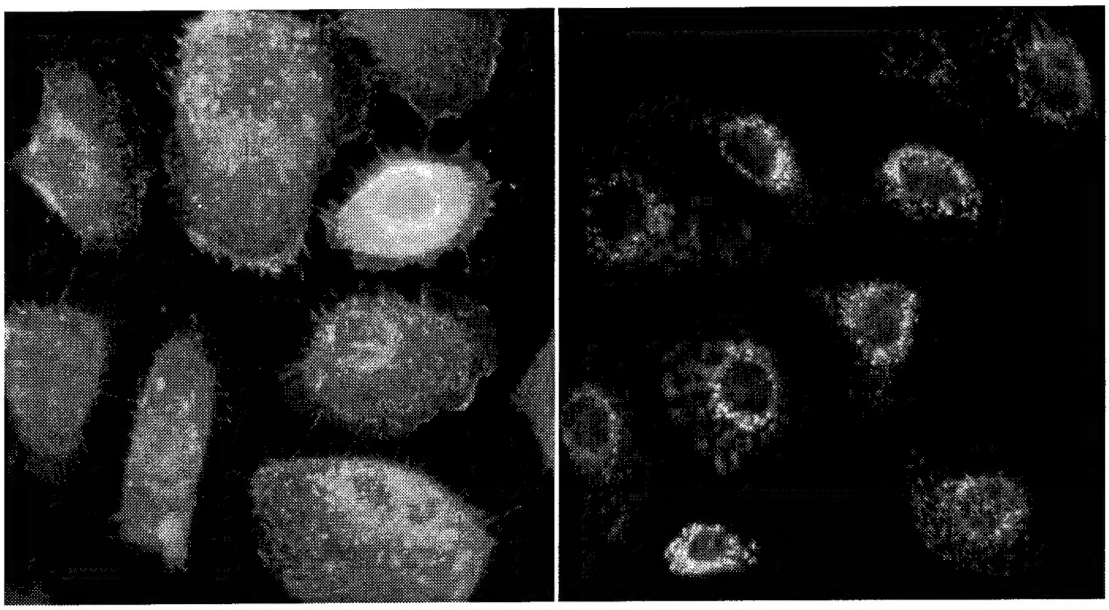

Figure 1. Redistribution of the EGF receptor following ligand addition. Cells were plated on coverslips and incubated in the absence (left) or presence (right) of 100ng/ml EGF for 120 min prior to fixation, permeabilization and staining with and anti-EGF-R monoclonal antibody followed by a FITC-conjugated secondary antibody. Photo taken with a 40X objective.

We have also succeeded in localizing the EGF-R expressed in polarized cells. Shown in Figure 2 is the distribution of both EGF-R and ZO-1 of either MDCK cells or 184A1 cells grown as monolayers on Transwell filters. This data indicates that 184 HMEC do not form well differentiated polarized epithelial layers on membrane. Because of this observation, we have begun work on the more differentiated HB2 cells (see above).

Work is also continuing on immunolocalization of EGF-R in frozen thin sections of organoid structures. However, the fine structure of these in-vitro formed structures is not sufficient to provide appropriate landmarks to interpret the spatial distribution of the receptors. In addition, there is a dramatic loss of EGF-R in these structures that makes it difficult to detect their presence (see below). Further work is required to determine which cell type is most appropriate for these studies. In the meantime, we continue to work with Transwell cultures because they are a more reproducible experimental system.

B. Kinetic analysis of the synthesis and turnover of the EGF receptor will be done. These studies have essentially been completed and are currently being written up for publication. We found by standard pulse-chase analysis that the half-life (turnover) of the unoccupied EGF-R was approximately 7hrs (Figure 3). Upon occupancy, this decreased to about 1.5 hr. This is a fairly typical response of cells to occupancy of the EGF-R. What was not expected, however, was the fact that the internalization rate was not altered by occupancy.

In every other cell type described to date, occupancy of the EGF-R results in induced internalization of the receptor. As shown in Fig. 4, however, the internalization rate of the occupied EGF-R is the same as the unoccupied EGF-R in 184A1 cells. This is due to a very rapid constitutive internalization rate. Other mammary epithelial cell lines show the typical

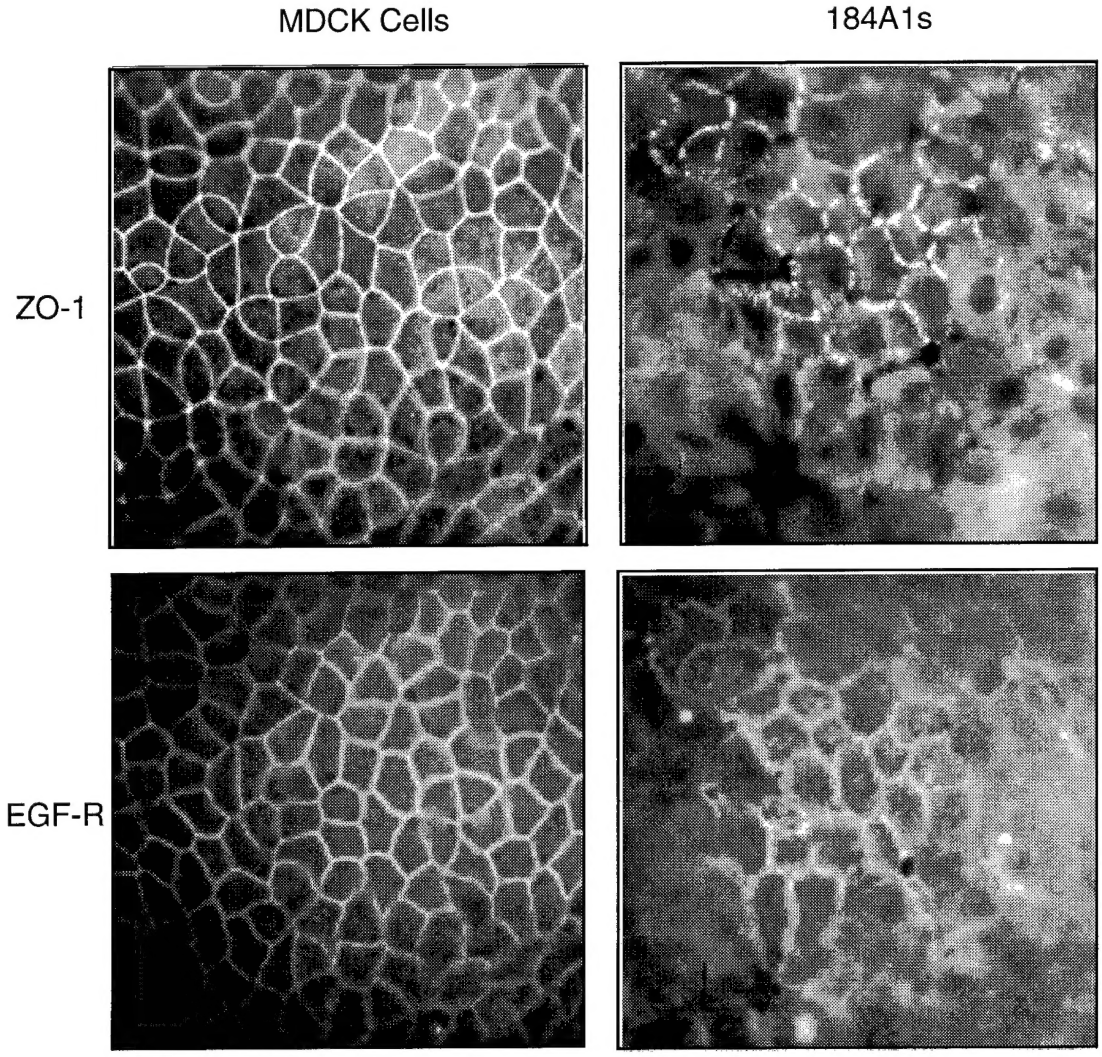

Figure 2. Distribution of the marker ZO-1 and the EGF-R on epithelial cells grown on Transwell filters. The indicated cells were grown to confluency and then fixed and stained with a rabbit antibody to ZO-1 (top panels) or a mouse monoclonal to the EGF-R (bottom panel). The two markers were visualized simultaneously with FITC and Texas Red, respectively.

pattern of a low constitutive internalization rate and high induced internalization rate. This data suggests that the constitutive internalization rate of the EGF-R is a regulated process. It also suggests that the increased degradation rate of the occupied EGF-R in 184 cells (Fig. 3) is not due to induced internalization, but instead to induced lysosomal targeting. This may have significant implication with respect to the relationship between HER1 and HER2/neu and breast cancer. For example, we recently have found that HER2 appears to be involved in targeting the EGF-R (HER1) to the lysosomes. Mutations in HER2 could therefore disrupt normal EGF-R downregulation in these cells, contributing to the transformed phenotype.

Because of these observations, we have accelerated our planned studies on disruption of

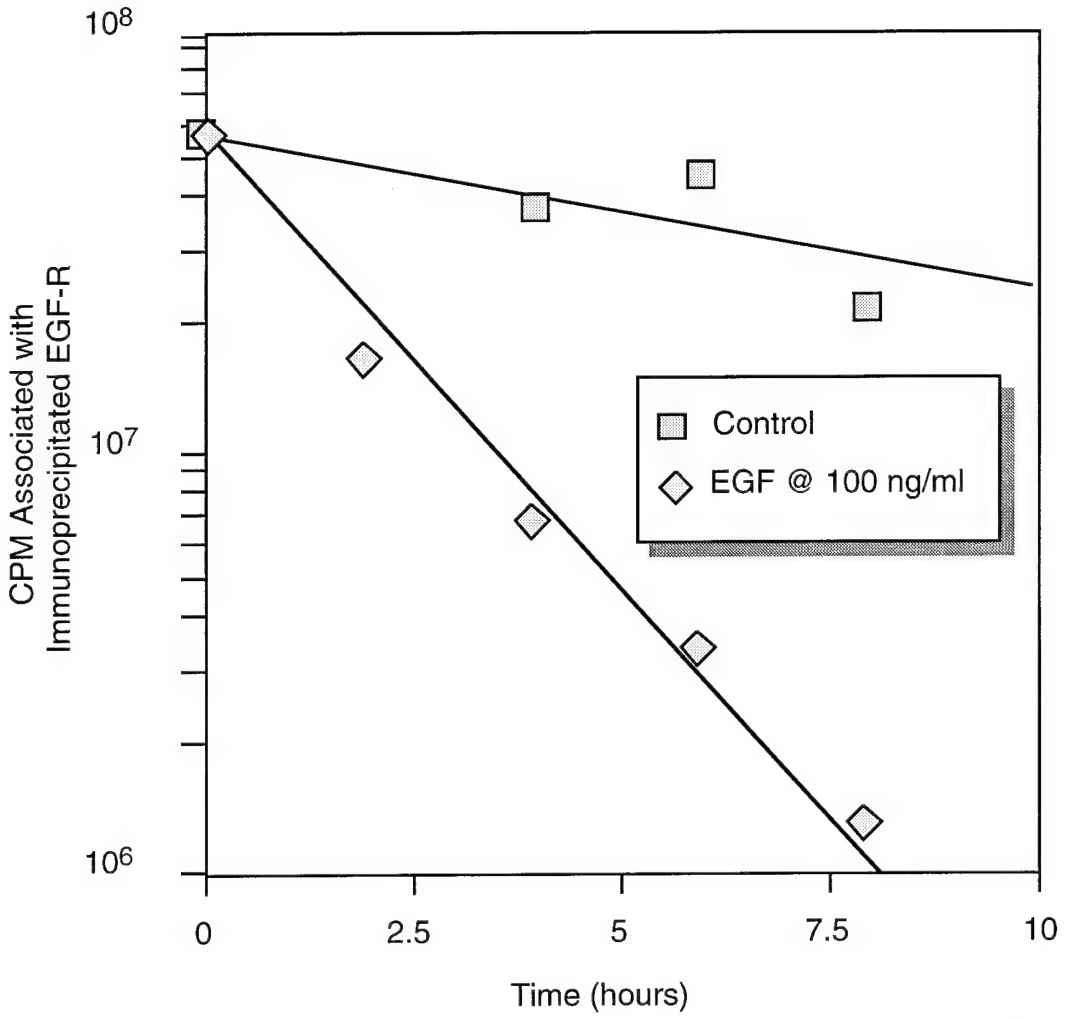

Figure 3. Turnover of the EGF receptor in HMEC is accelerated by the addition of EGF. Cells were labeled overnight with [^{35}S]methionine and then chased for the indicated lengths of time in either the presence or absence of EGF. The cells were extracted and receptors were isolated by immunoprecipitation and gel electrophoresis. The amount of label remaining in the receptor bands was then determined using a phosphoimager.

EGF-R trafficking by introducing dominant-negative receptor trafficking mutants (see Task 3 above). Two such mutants have been prepared. One has already been introduced into HMEC and the other mutant has been introduced into fibroblast test lines (to establish their dominant-negative phenotype). These studies should be completed in the next year.

C. The level at which the EGF receptor is regulated in normal HMEC will be determined.
As discussed above, our studies suggest that the EGF-R is regulated at the level of lysosomal targeting in 184 cells. However, other human mammary epithelial cell lines (such as HB2 cells) are regulated at the level of both internalization and lysosomal targeting (Fig. 4). Because 184 cells have a "basal" phenotype whereas the HB2 cells (and most breast cancer lines) have a "luminal" phenotype, a transition from one regulatory system to another may occur during differentiation in this cell type.

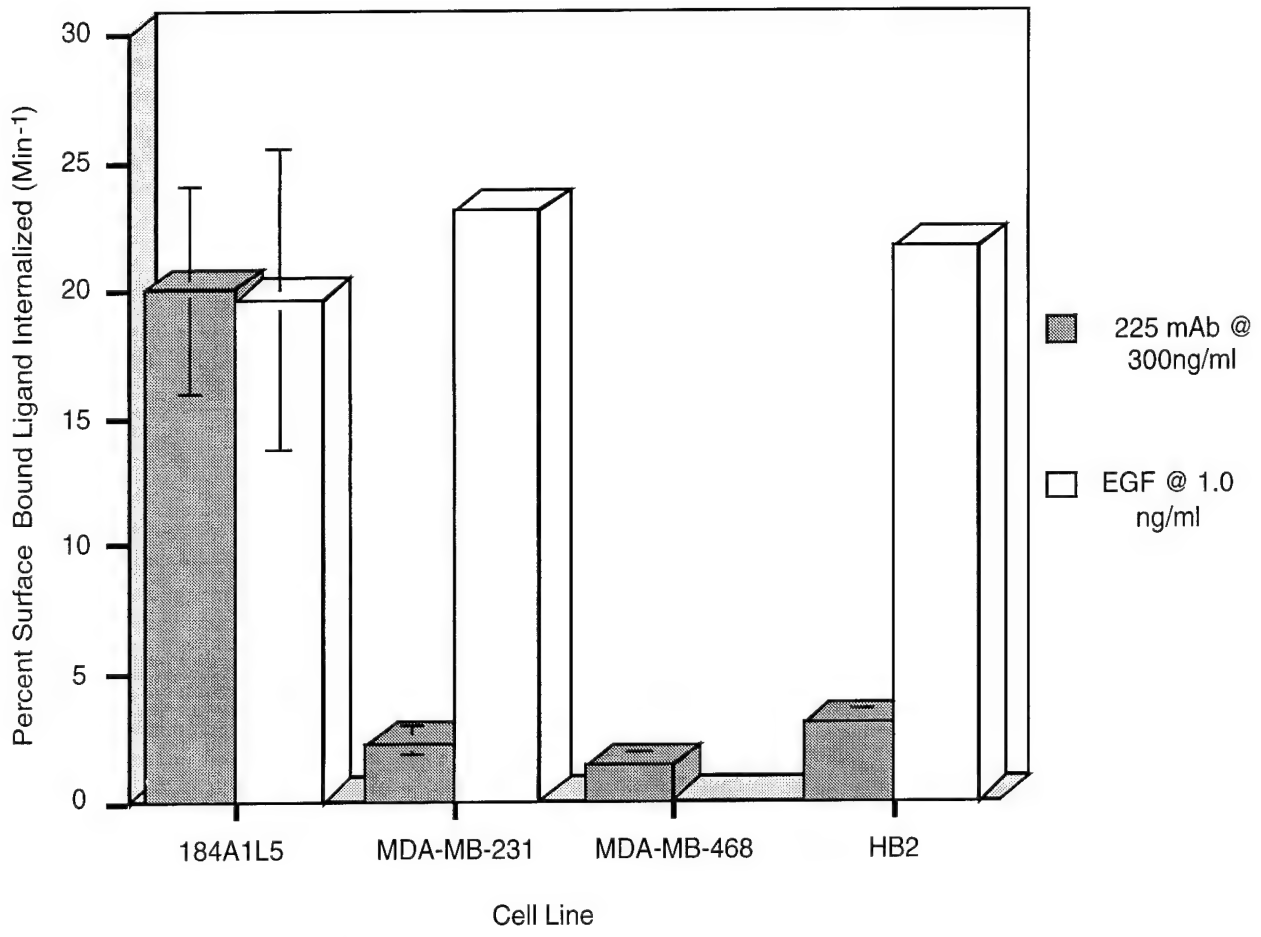

Figure 4. Rapid constitutive internalization rates of empty EGF receptors in 184A1 cells. The internalization rates of occupied receptors was determined using radiolabeled EGF whereas empty receptor were analyzed using labeled antagonistic antibody 225. Specific internalization rates were determined by internalization plot analysis as previously described (39).

There are other potential levels of receptor regulation other than a simple change in receptor mass. In particular other studies in our laboratory suggest that overexpression of EGF-R is associated with a loss of receptor desensitization. To pursue this line of investigation, we have developed a series of novel ELISA assays to measure EGF-R mass and extent of tyrosine phosphorylation. These assays are being used to determine whether the ability of HMEC to desensitize the EGF-R is normal. A paper describing this novel assay and results obtained from cells overexpressing EGF-R is currently being prepared for submission.

D. The levels of regulation which are modulated by changes in cellular environment will be established. These studies are currently being completed using the internalization assays (see above) and western blot analysis. One of the most interesting findings is that EGF-R expression is decreased when cells are cultured on matrigel (Figure 5). Our current working hypothesis is that this is due to a decrease in receptor mRNA (see subtask E). The possibility that extracellular matrix can modify internalization rates or lysosomal targeting rates are being pursued, but are not yet completed.

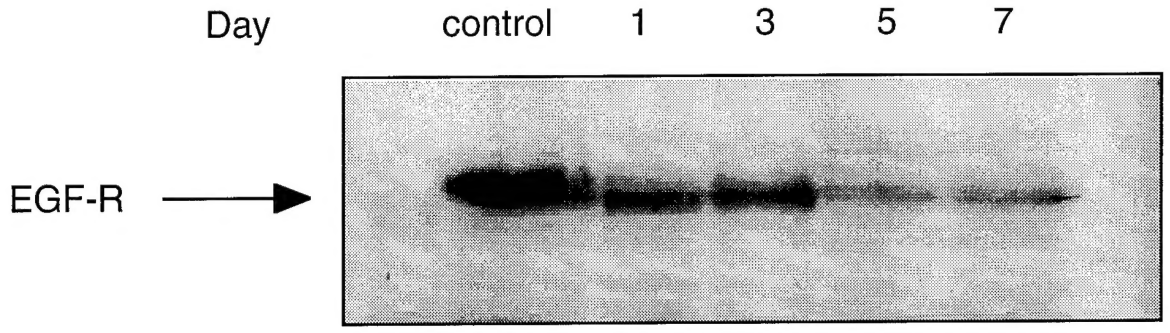

Figure 5. Decreased EGF-R expression during organization on matrigel as determined by western blot analysis. Equal amounts of cell protein were loaded in each lane; the control is cells grown on plastic. The level of receptor protein was evaluated by probing to an anti-EGF-R antibody.

E. Using quantitative PCR, the changes in ligand and receptor expression during formation of alveolar structures will be determined. Mammary epithelial cells will spontaneously form organized structures when cultured on matrigel. To better define the regulation of EGF-R and it ligands during organization, we defined the change in both protein and mRNA expression on matrigel as well as selected components of matrigel.

To understand the level at which this regulation was occurring, quantitative PCR was done on HMEC grown on thin layers of matrigel as well as matrigel components. As shown in Fig. 6, there is a significant reduction in EGF-R mRNA expression when cells are cultured on matrigel (relative to the internal G3PDH standard). In addition, ligand expression is also significantly reduced. The component of matrigel responsible for reduction of EGF-R expression appears to be laminin (Fig. 6). One interesting aspect about the data in Fig. 6 is that the HMEC express 4 different ligands for the EGF-R (TGF-α, AR, HB-EGF and EGF). The pattern of expression of these different ligand is also modulated by the particular matrix on which the cells are grown.

One aspect of regulation not addressed by simple analysis of mRNA levels is whether changes in synthetic rates are accompanied by changes in secretion rates. The different ligands for the EGF-R are known to be synthesized as transmembrane precursors, the cleavage of which is a regulated process. If a particular ligand is not cleaved, it will be internalized and presumably targeted to lysosomes for degradation. Because of the striking changes in expression of AR and TGF-α by HMEC grown on different matrices, it is important to determine whether their release from cells is also regulated. Towards this end, we have created several chimeric ligands that contain the different sequences that presumably regulate the release of the different ligands. These artificial ligands will be used as reporter molecules to follow the activation of the enzymes involved in ligand release. Theses studies have been initiated and should be completed in the next year.

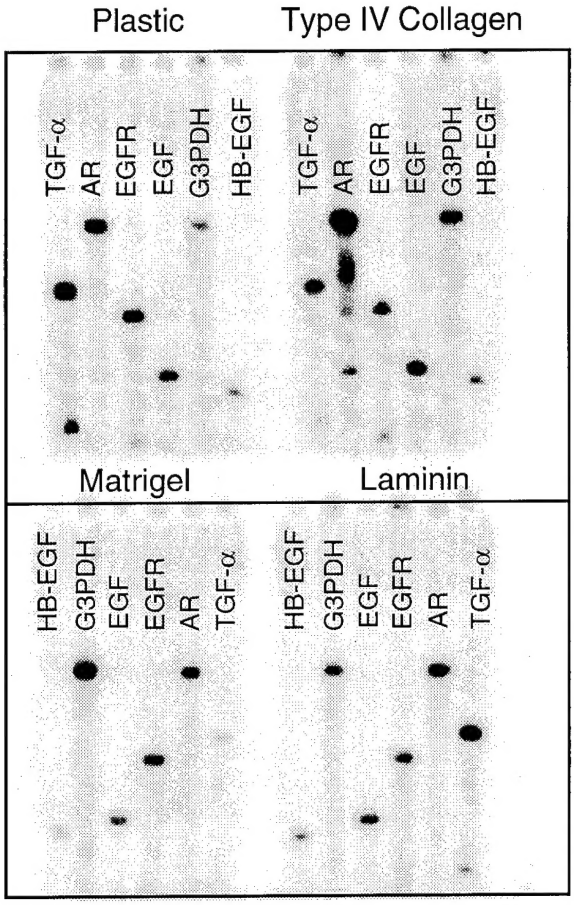

Figure 6. Relative expression of EGF-R and its ligands in HMEC cultured on different extracellular matrices. 184A1 cells were plated in dishes precoated with the indicated matrices and grown for 3 days. Cellular mRNA was extracted and subjected to RT-PCR using the appropriate primers and standard techniques. The labeled reaction products were separated on acrylamide/guanidine gels and visualized using a phosphoimager.

CONCLUSIONS

Our studies are completely on target. As expected, the first year was primarily used to develop our experimental systems and to establish appropriate assays. This has been accomplished. Once we verify that the HB2 cells display a polarized EGF-R distribution, we will use them to start the studies on looking at the effect of oncogene disruption on EGF-R distribution. Because of our discoveries on the importance of ligand distribution and receptor trafficking to receptor down regulation, we will devote more attention to this phase of our studies for the coming year.

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